nanodiscs is accomplished by its addition to the initial lipid and MSP mixture. Microscale thermophoresis (MST) is a recently developed technology that studies interactions based on the differential movement of biomolecules and their complexes in a microscopic temperature gradient. MST is highly sensitive, since it depends on changes in the size, charge and hydration shell of molecules. It measures interactions in free solution (no coupling required) and needs only very low sample volumes. It thus provides advantages over existing techniques like fluorescence correlation spectroscopy (FCS), isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). The use of nanodiscs in MST-based interaction studies of membrane proteins with ligands, or of lipid membranes with soluble peptides is very appealing. We thus explored this approach experimentally. Potential pitfalls of the method (aggregation of nanodiscs, undesired interactions with the capillary walls, etc.) were addressed and ways to overcome such difficulties are presented. Finally, MST was successfully applied for the measurement of binding affinities between various membrane proteins and their ligands.

2863-Pos Board B18

Design and Testing of High-Affinity Mutants of Interferon Gamma Receptor 1

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Specific protein-protein interactions control many crucial processes of the living cell. We aim at elucidating specificity of the interactions at the model system of interferon gamma (IFNg) and its cellular receptor 1 (IFNgRec1), the system important in innate immunity. To modulate (increase as well as decrease) specificity of the interaction we searched for mutations of the receptor molecule that was subjected to in silico mutations using the crystallographically determined structures of IFNg/IFNgRec1 complex. Amino acid substitutions were modeled by empirical force field implemented in the web-based software FoldX. About twenty computer-selected candidate mutants of IFNgRec1 were successfully expressed in Escherichia coli, purified to homogeneity and their affinities to IFNg were determined by surface plasmon resonance (SPR). The SPR measurements showed that affinity of most receptor variants designed for affinity increase had their affinity virtually unchanged, a few had affinity slightly lower but a few bound with affinity significantly higher. Simple and computationally cheap method was therefore able to predict increase of affinity. The receptor variants with increased affinity may be used for diagnostic purposes.

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2864-Pos Board B19

Investigating a Benzofurane Derivative Binding Site on Human ClC-5

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Human ClC-5 is a Cl^-/H^+ antiporter belonging to the CLC family, which includes both Cl^- channels and Cl^-/H^+ antiporters. Small organic molecules have been useful tools for studying ion channels and many commercial drugs target specific ion channel proteins. Some blockers have been found to inhibit the plasma membrane localized CLC channels ClC-0, ClC-1 and ClC-Ka. However, high-affinity ligands for most CLCs are missing.

Here, we tested the benzofuran derivative RT-93[1,2] on ClC-5 and ClC-4. Interestingly RT-93 inhibits ClC-5 (EC50 ~ 200 μM), but has only a small effect on the highly homologous antiporter ClC-4. Towards our goal to identify the binding site of RT-93 on ClC-5, we constructed 12 chimeras between ClC-4 and ClC-5. Guided by the crystal structure of a bacterial CLC protein and using the alignment between ClC-4 and ClC-5, we identified the sequence stretches with the main differences between these two proteins and substituted in ClC-5 the portions with the corresponding sequence of ClC-4. We expect to remove the RT-93 sensitivity at least in one of these. So far we tested 8 chimeras, none of which, however, showed a significant difference in comparison with ClC-5. If none of the chimeras eliminates the binding site we will proceed with constructing larger chimeras.

Discovering the binding site of this blocker might help in creating a more efficient ligand that, apart from being a useful biophysical tool, may provide a way to modulate protein function *in vivo.*

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2865-Pos Board B20

Cloning, Expression, and Purification of a Centrin Biological Target: Krr1, KH Domain

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The origin of many disease states has been linked to genetic mutations, defects in gene expression, nuclear excision repair and ribosome biogenesis. Centrin, a calcium binding protein, which has recently been found to regulate some of these processes along other target proteins within the nucleus. One target, Krr1, contains a K homology (KH) domain; which has been identified as a nucleic acid recognition motif, required for proper processing of pre-rRNA, for synthesis of 18S rRNA, and for the assembly of the 40S subunit. Our initial findings have identified a putative centrin binding site located within the KH domain of Krr1 using bioinformatics tools. In this study, the KH domain (192 bp) was amplified by PCR and then ligated to the expression vector pET100. Colony PCR was performed to identify the E. coli colonies that have been transformed effectively with the desired recombinant. The Krr1 KH domain fragment was then overexpressed. An isolation and purification protocol has been designed which includes preparative centrifugation, cross flow and tangential flow filtration and two ion exchange chromatography. The evaluation of the purification of the KH domain fragment has been performed by SDS-PAGE and peptide samples have been sent for MS analysis and partial amino acid sequencing.

2866-Pos Board B21

Recognition of Mesothelin by the Therapeutic Antibody MORAB-009: Structural and Mechanistic Insights

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Mesothelin is a cell surface protein that is normally found in mesothelial cells lining the pleura, pericardium and peritoneum, but is aberrantly expressed at high level in a variety of cancers including mesothelioma, ovarian, pancreatic and lung cancers. Although the physiological function of mesothelin is unclear, studies have shown that it is capable of binding to the tumor antigen CA-125 (also known as MUC16), leading to cell adhesion and tumor metastasis. Since mesothelin is specifically expressed at a significantly higher level in malignant tumors, development of an antibody against mesothelin is therefore of major importance in the field of cancer therapy. MORAb-009 is a humanized monoclonal antibody against mesothelin currently under clinical trials. Animal experiments have shown that application of MORAb-009 in combination with chemotherapy leads to a marked reduction in tumor growth of mesothelin-expressing tumors.

We show here that MORAb-009 recognizes a non-linear epitope that is contained in the first 64-residue fragment of the mesothelin. We further demonstrate that the recognition is sensitive to the loss of a disulfide bond linking residues Cys7 and Cys31. The crystal structure of the complex between the mesothelin N-terminal fragment and Fab of MORAb-009 at 2.6 Å resolution reveals an epitope encompassing multiple secondary structural elements of the mesothelin, The mesothelin fragment has a compact, right-handed superhelix structure consisting of five short helices and connecting loops. A residue essential for complex formation has been identified as Phe22, which projects its side chain into a hydrophobic niche formed on the antibody's recognition surface upon antigen-antibody contact. The overlapping binding footprints of both the monoclonal antibody and the cancer antigen CA125 explains the therapeutic effect and provides a basis for further antibody improvement.

2867-Pos Board B22

Design, Synthesis, Binding, Crystallography, and Docking of [(2-Pyrimidinylthio)Acetyl]Benzenesulfonamides as Inhibitors of Human Carbonic Anhydrases

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A series of [(2-pyrimidinylthio)acetyl]benzenesulfonamides were designed and synthesized. their binding affinities as inhibitors of several recombinant human carbonic anhydrase (CA) isozymes were determined by isothermal titration calorimetry and thermal shift assay yielding intrinsic Gibbs free energies, enthalpies, entropies, and heat capacities of binding. A group of compounds containing a chlorine atom in the benzenesulfonamide ring were found to exhibit higher selectivity but lower binding affinity toward tested CAs. The crystal structures of selected compounds in complex with several CAs were determined and the docking studies were performed to compare the binding

modes of experimentally determined crystallographic structures with computational prediction of the pyrimidine derivative binding to CAs. Structure-thermodynamics correlations will be discussed. Several compounds bound to select CAs with single-digit nanomolar affinities and could be used as leads for inhibitor development towards anticancer target CA isozymes.

Heme Proteins

2868-Pos Board B23

Structural Dynamics of the Signal Transducer Protein HemAT as Revealed by Time-Resolved Step Scan FTIR Spectroscopy

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Aerotaxis is an important biological process for bacteria, as they utilize a signal transduction system to rapidly sense and respond to fluctuating O_2 levels and retain their homeostasis. HemAT isa heme-based O_2 sensor protein that acts as a signal transducer for aerotaxis in *Bacillus subtilis*, controlling the organisms' movement towards increasing O_2 gradients. The molecular mechanisms for intra- and inter-molecular signal transduction processes are largely unknown. In our work, we have employed time-resolved step-scan Fourier transform infrared (TRS^2-FTIR) spectroscopy to investigate the protein structural changes induced by ligand (CO) photodissociation and rebinding that are crucial for understanding the initial events of the intramolecular signal transduction mechanism in HemAT. We have studied the truncated sensor domain and full length HemAT-CO adducts as well as the Y70F, Y133F, L92A, and T95A mutants. Monitoring the kinetics of CO rebinding to the heme-Fe^{2+} (t_d=6μs-6ms) reveals biphasic kinetics for both the full length and truncated sensor domain HemAT. The TRS^2-FTIR experiments have additionally revealed that Y70 predominantly controls the conformational changes that are induced to the protein matrix by CO photodissociation. Moreover, L92 appears to operate as the conformational gate in the migration pathway of photodissociated CO.

2869-Pos Board B24

Dynamic Allosteric Mechanism of Modulation of Oxygen-Affinity in Human Hemoglobin

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The O2-affinities of deoxy- and oxy-hemoglobin (Hb) [KT and KR,] are reduced as much as 102- and >103-folds, respectively, upon binding of potent heterotropic effectors(1). However, the quaternary structures, the stereochemical structures of the heme environment, the heme coordination structures and the axial electronic structures of the heme coordination (1/2 Fe-His = 215 cm-1 or 1/2 Fe-O = 567cm-1)(3), either in crystal(2) or in solution(1,3), show no detectable changes upon binding of these potent heterotropic effectors. Thus, the O2-affinities of Hb [KT and KR] are correlated to neither T/R-quaternary structures nor the ligation states of Hb in the presence and absence of heterotropic effectors. They, further, indicate that these effector-bindings to either deoxy- or oxy-Hb do not alter the O2-affinities of the hemes in the respective ligation states of Hb, though they reduce the O2-affinities of the respective states of Hb. Our molecular dynamics simulation(4) indicates that the amplitudes of high-frequency thermal fluctuations of the protein matrix significantly increase as the O2-affinity of Hb is reduced, independent of the quaternary structures and the ligation states. Bi-molecular ligand association/dissociation processes to/from the hemes in hemoproteins such as Mb and Hb are interfered from physical barriers of the protein matrix (the ''Cage'' effect). The O2-affinity in Hb is regulated by the effector-linked, dynamic modulation of high-frequency thermal fluctuations of the protein matrix in Hb (the dynamic allostery mechanism) rather than static quaternary/tertiary structural changes in Hb.

References:
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2870-Pos Board B25

Roles of Amino Acid Residues in Woolly Mammoth Hemoglobin on the Temperature Effect of Oxygen Binding

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The O_2 affinity of hemoglobin (Hb) is affected by allosteric effectors (e.g. H^+, chloride, and organic phosphate) and temperature. Hb oxygenation is exothermic, i.e., the oxygen-binding affinity increases significantly with decreasing temperature. This makes it difficult for the Hb molecule in blood to deliver oxygen to the tissues under conditions of hypothermia during major surgical operations. We constructed plasmids to express recombinant woolly mammoth Hb (rHb WM) and Asian elephant Hb (rHb AE). Our biochemical-biophysical studies show that the apparent heat of oxygenation (ΔH) of rHb WM is less negative than that of rHb AE and human normal adult Hb (Hb A), suggesting that the O_2 affinity of rHb WM is much less dependent on temperature. In order to investigate the key residues of the Hb molecule responsible for the temperature effect on O_2 affinity, mutants with β/δ101 substitutions (β/δ101Gln→ Glu, Lys, and Asp) in rHb WM have been expressed.Compared to rHb WM,these mutants exhibit a higher affinity for oxygen, and a more negative ΔH value under various conditions of pH, temperature, and salt concentration, with and without organic phosphates. Titrations for the O_2 affinity of those mutant rHbs as a function of chloride concentrations indicate a lower heterotropic effect of this anion due to the replacement of β/δ101Gln, suggesting that the β/δ101Gln residue in rHb WM is important for its stronger response to chloride ions, and also responsible for its lower temperature effect of O_2 affinity. NMR measurements for rHb WM and its mutants have been used to correlate their structural and functional properties. These findings could provide new insights into designing hemoglobin-based oxygen carriers (HBOCs) for treating patients undergoing therapeutic hypothermia (e.g. cardiac arrest, traumatic brain injury, stroke, etc.).

2871-Pos Board B26

Time-Resolved Thermodynamics and Transient Kinetics for Oxygen Photorelease from Hemoglobin:Effector Complexes

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Photoacoustic calorimetry and transient absorption have been used to characterize thermodynamic and kinetic parameters for oxygen photorelease from human hemoglobin (Hb) complexed with allosteric effectors in terms of time-resolved volume and enthalpy changes, quantum yields for bimolecular rebinding, and rate constants for oxygen rebinding. Under stripped conditions we observe a significant temperature dependence of enthalpy and volume changes associated with oxygen photorelease. Below 16° C an endothermic enthalpy and volume expansion ($\Delta H = 272 \pm 60$ kcal mol^{-1}, $\Delta V = 19.5 \pm 4.5$ mL mol^{-1}) were determined for O_2 photorelease, while above 16° C the reaction becomes exothermic and a volume contraction is observed ($\Delta H = -252 \pm 79$ kcal mol^{-1}, $\Delta V = -58.3 \pm 16.5$ mL mol^{-1}). Similar temperature dependence of $\Delta V/\Delta H$ was observed in phosphate buffer at ionic strength of up to 0.2 M. Moreover, the reaction volume and enthalpy changes observed in the presence of 500 mM NaCl (I = 0.5 M) are significantly smaller ($\Delta H = -27 \pm 8$ kcal mol^{-1}, $\Delta V = 7.9 \pm 0.7$ mL mol^{-1}) and the temperature dependence is eliminated, suggesting the significant contribution of electrostriction to the observed reaction parameters. L35 also eliminated the temperature dependence without affecting volume and enthalpy changes associated with O_2 photorelease, effectively extending the low temperature trend. L35 is known to bind strongly in the central cavity of Hb in the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ clefts, and may exhibit this effect by modulating intersubunit interactions. IHP acted similarly but decreased related enthalpy changes. In addition, allosteric effectors and phosphate buffers were found to increase the quantum yield for bimolecular rebinding of oxygen, and rate constants for oxygen rebinding to Hb:L35 and Hb:IHP complexes were lower than those for the stripped protein.

2872-Pos Board B27

Oxygen-Dependent Depolymerization of Sickle Cell Hemoglobin Polymers in the Lungs: Kinetic Mechanisms and their Significance for Pathogenesis and its Prevention

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Our kinetic studies have characterized the mechanisms of deoxyhemoglobin S polymer depolymerization when exposed to CO (used as a model for oxygen because rates can be controlled photolytically). Fibers dissolve slowly, losing monomers from their ends at low partial pressure, and very rapidly at higher partial pressures that induce fiber fracture and therefore many new ends. Slow dissolution that is not complete in the time red cells traverse the pulmonary microvasculature will generate residual arterial polymers (RAPs), enhancing pathogenesis by seeding nucleation of new polymers, accelerating repolymerization and increasing its extent.